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CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO THE
NEBRASKA STRAIN OF BOVINE CORONAVIRUS

BY

JEANIE MARIE (NELSON) LEMBKE

A thesis submitted in partial fulfillment
of the requirements for the degree
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CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO THE
NEBRASKA STRAIN OF BOVINE CORONAVIRUS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by this candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

ATCC--American type culture collection
BCV--Bovine coronavirus
C--Celsius
CCV I-71--Canine coronavirus strain I-71
CPE--Cytopathic effects
CRFK--Crandell feline kidney cells
ELISA--Enzyme-linked immunosorbent assay
FBS--Fetal bovine serum
FFN--Fluorescent focus neutralization
FIPV--Feline infectious peritonitis virus
HAI--Hemagglutination inhibition
HCV-229E--Human coronavirus strain 229E
HCV-OC43--Human coronavirus strain OC43
HEV-67N--Hemagglutinating encephalomyelitis virus
strain 67N
IBV--Infectious bronchitis virus
IEM--Indirect immunoelectron microscopy
IFA--Indirect fluorescent antibody
IgG₁--Subclass of immunoglobulin G
IgG_{2a}--Subclass of immunoglobulin G
Mab--Monoclonal antibody
MEM--Minimal essential medium
MHV--Mouse hepatitis virus

NS-1--Myeloma line of Balb/c origin that does not
synthesize heavy chains of an immunoglobulin

P3-X63-Ag8.653--Myeloma line of Balb/c origin that
does not synthesize light or heavy
chains of an immunoglobulin

PBS--Phosphate buffered saline

RPMI--Roswell Park Memorial Institute media

SP2/OM1--Myeloma line of Balb/c origin

ST--Swine testicle cells

TCID₅₀--Tissue culture infective dose

TCV--Turkey coronavirus

TGE--Transmissible gastroenteritis virus

v/v--Volume/volume

VN--Viral neutralization

w/v--Weight/volume

SUMMARY

The purpose of this study was to develop and characterize a panel of monoclonal antibodies specific for epitopes on the Nebraska strain of bovine coronavirus (BCV). The hybridomas were prepared by fusion of spleen cells from BALB/c mice hyperimmunized with BCV and from SP2/OM1, NS-1, and P3-X63-Ag8.653 myeloma cell lines. Seven hybridoma clones were derived from the fusions. The immunoglobulin isotype IgG1 was the most common antibody produced by the hybridomas. BALB/c mice were inoculated with hybridoma cells to produce ascitic fluids. Assays were performed on the ascitic fluids to determine which biological functions of the bovine coronavirus virions were identified by each of the seven monoclonal antibodies. One of the seven monoclonal antibodies (25-48) neutralized BCV, one (4-7) inhibited hemagglutination, while the remaining five reacted on indirect immunofluorescence and enzyme-linked immunosorbent assays.

All monoclonal antibodies reacted with BCV on the indirect immunofluorescence assay and titers ranged from 1:10 to 1:3200. Three (71-44, 6-1, and 6-4) of

the seven monoclonal antibodies also reacted on indirect immunofluorescence assays with hemagglutinating encephalomyelitis virus (HEV) of swine, which is antigenically related to BCV. These three monoclonal antibodies are directed against an epitope common to both BCV and HEV.

Hybridoma 4-7 produced monoclonal antibody that inhibited hemagglutination of rat erythrocytes by BCV, and therefore, recognized epitopes common to the viral hemagglutinin. Clone 25-48 produced monoclonal antibody which neutralized BCV at titers ranging from 1:32 to 1:1024. The observation that Mabs which prevented hemagglutination independent of neutralization suggested that either these functions were on separate glycoproteins of the virion or the hemagglutinin and neutralization epitopes are separate regions on the same glycoprotein. The latter hypothesis was suggested by the immune electron microscopy results. When BCV was treated with bromelain, most of the outer row of surface projections were removed and the antigen (BCV)-antibody (25-48) reaction observed on immune electron microscopy was enhanced. Since bromelain has been reported to remove all glycoproteins except the hemagglutinin (33),

the enhanced reaction between Mab 25-48 and BCV on immune electron microscopy suggests that the neutralizing Mab 25-48 reacted with the viral hemagglutinin. Further characterization of the monoclonal antibodies obtained in this study by Western blotting or immunoprecipitation will be required to clearly define the molecular weights of the proteins to which the monoclonal antibodies react.

In summary, all of the monoclonal antibodies, 7/7, had immunofluorescent titers (IFA) against BCV, but 3/7 (71-44, 6-1, and 6-4) also reacted with HEV which indicates that these antigenically related viruses share a common viral protein. One monoclonal antibody (25-48) recognized a neutralization epitope, and one (4-7) recognized the virion hemagglutinin, suggesting these are separate epitopes on different glycoproteins or are epitopes on the same glycoprotein present on the virion surface. The latter hypothesis was favored by the immune electron microscopy observations, which reveal that the neutralizing antibody binds to the hemagglutinin of bromelain treated BCV.

INTRODUCTION

Bovine coronavirus (BCV) is an acute enteric disease of cattle, most often fatal in newborn calves (46,71,72) and widely prevalent since most adult cattle have antibody to this virus (54). The absorptive epithelial cells covering the small intestinal villi and the crypt cells of the colon are the main targets of the virus (41,46,71). The infection of these cells leads to villous atrophy, resulting in a malabsorptive diarrhea, dehydration, and often death (41,46). Affected herds of cattle with 100 percent morbidity and up to 50 percent mortality in susceptible calves are not uncommon (4).

Bovine coronavirus belongs to the Coronaviridae family, a group of enveloped viruses possessing a single stranded linear RNA genome of positive polarity (20,71). The bovine coronaviruses have a double row of surface projections or envelope peplomers, while other members of this family possess only a single row of peplomers (71). These peplomers are glycoproteins present on the surface of the virus and function as antireceptors (16,20,27,36), hemagglutinins (13,32), site of neutralization (13,14), and fusion proteins

(14). Four glycoprotein (gp) peplomers, 140, 120, 100, and 26K, have been identified on the surface of BCV (32). The 120-140K gp has been identified as the viral hemagglutinin by one group of investigators (32,33), whereas a second group identifies a 105K gp as the hemagglutinin (68). The functions of the other three surface gp of BCV have not been delineated. Within the mammalian coronaviruses, BCV is antigenically related to mouse hepatitis virus (MHV), hemagglutinating encephalomyelitis virus (HEV), and the human respiratory virus (OC-43) (71).

The precise function of virion proteins, such as the four surface glycoproteins of BCV, can best be determined by using monoclonal antibodies (Mabs). These Mabs are specific for epitopes of different virion proteins and can be used to determine the function of such proteins. The production of Mabs to coronaviruses has allowed the determination of function for surface gp of MHV (70), infectious bronchitis virus (IBV) (13), and transmissible gastroenteritis virus (TGE) (16). Two reports have described the development of Mab which neutralize and prevent hemagglutination of BCV (17,68). Some of these Mabs to BCV neutralized the virus, some prevented

hemagglutination, and others had both functions (17,68). Results of immunoprecipitation tests suggested that these Mabs precipitated either a gp 120-140 (17), or a gp 105 (68). These results have been confusing since King et. al. (33) have identified the gp 140 as the hemagglutinin. The gp 140 is analogous to the gp 120 described by Deregt et. al. (17), but the gp 105 is a gp on the outer row of projections (68). Thus the gp 120-140 or gp 105 may be the site of both neutralization and hemagglutination, or the sites may be separate since some Mabs neutralize but do not hemagglutinate (17,68). Presently, the site of the BCV hemagglutinin and neutralization have not been definitely identified.

The purpose of the present study was to develop and characterize a panel of Mabs to the Nebraska strain of BCV. The aim of the study was to obtain information on the function of the virion proteins with the hope that these Mabs could eventually be used to improve diagnosis and detection of the BCV. The general biological properties of seven hybridomas, two directed against surface gp and five against internal viral proteins, are discussed.

LITERATURE REVIEW

The family Coronaviridae are classified on the basis of similar morphological, biochemical, and biophysical properties (58,59,60). Coronaviruses are pleomorphic particles 60 to 200 nm in diameter (59,60,71), and possess a linear genome of single-stranded RNA which is positive-stranded, infectious, polyadenylated (21,58,71), and has a molecular weight of $5-7 \times 10^6$ (58). Coronaviridae are the only recognized viruses with a single-stranded, positive polarity, RNA genome with nucleocapsids of helical symmetry surrounded by a lipid envelope (21). The lipid envelope is obtained as nucleocapsids bud into the lumen of the rough endoplasmic reticulum (21,27,58,71), or Golgi apparatus (27). The lipid envelope is biochemically and structurally identical to the host cell membranes from which it is derived (51). Chemically, the envelope contains neutral lipids, phospholipids, and glycolipids (21) and consists structurally of a lipid bilayer (21,71). Prior to budding of the nucleocapsids, viral proteins are inserted into the membranes of the rough endoplasmic reticulum and the Golgi apparatus (60).

Enveloped viral particles, migrate through the Golgi apparatus and are packaged into secretory vesicles. These vesicles fuse with the plasma membrane and the virions egress from intact cells, but do not bud from the plasma membrane (27). Thus, coronaviruses may be released from cells by utilizing a cellular transport mechanism developed for secretion or exocytosis of the contents of secretory vesicles (63,64). Enveloped, extracellular virions have a fringe of club-shaped surface projections or peplomers, which are 20 nm in length and surround the virus particle (59,71). These peplomers give the characteristic morphology of a "solar corona" or "crown" from which the family name is derived (63).

Coronaviruses have three major classes of virus proteins, the surface projection (peplomer) protein, nucleocapsid protein, and the transmembrane (matrix) protein. The peplomer protein is a glycosylated polypeptide (gp) of 80,000 to 200,000 molecular weight (58,60). The peplomer protein functions as the viral attachment protein or antireceptor, which binds to the receptor on the plasma membrane of the host cell (20). It also functions in cell fusion, and, in some strain of virus, is the hemagglutinin (14,58).

Monoclonal antibodies prepared against the peplomer proteins neutralize viral infectivity suggesting that the peplomer proteins are the site of viral neutralization. The peplomer proteins may be removed by treatment with proteases such as bromelain or by growing the virus in cell cultures with media containing tunicamycin, an antibiotic which inhibits glycosylation (32,58). Use of either proteases or tunicamycin results in virions lacking peplomers and these viruses do not bind receptors on the cell surface or initiate infection (27).

The nucleocapsid protein is a nonglycosylated protein of 50,000 to 60,000 molecular weight (60). This protein is surrounded by the lipid envelope, which protects the nucleocapsid from proteases such as bromelain (32,63). This protein interacts with newly formed viral RNA to form the ribonucleoprotein of the viral nucleocapsid (64). Phosphorylation of nucleocapsid proteins has been demonstrated for bovine coronavirus (BCV) (32,58), infectious bronchitis virus (IBV) (37,58), and MHV (58,62). The function of nucleocapsid protein phosphorylation is unclear, although interaction between nucleocapsid ribonucleoprotein and the viral matrix protein may be

facilitated by phosphorylation of proteins during virus assembly (60). It has also been reported, in studies using a mouse coronavirus, that the degree of nucleocapsid protein phosphorylation regulates the interaction of the capsid proteins with the viral RNA. This interaction would then influence or regulate the formation of viral nucleocapsids (62).

The transmembrane (matrix) protein is a glycosylated polypeptide (gp) of 20,000 to 35,000 molecular weight (58,60). The glycosylated region of this protein is exterior to the virion envelope (59), while the remainder of the protein is partially concealed within the viral envelope and on the inner surface of the envelope (64). When treated with proteases, such as bromelain, the matrix protein is slightly reduced since the portion of the gp exterior to the virion envelope is removed after treatment (20,63). The protease resistant portion of the transmembrane protein is located on the internal side of the viral envelope (63). Monoclonal antibodies directed against the matrix protein of MHV do not neutralize the virus (14). The lack of neutralization by monoclonal antibody to the matrix gp suggests that the epitope for neutralization of coronavirus is not

located on the matrix protein and this would be expected since this protein is not as easily accessible to antibody as are the surface projections. The matrix protein of MHV and other coronaviruses is responsible for determining conformation of the virion and designating the site of nucleocapsid budding (27).

Coronaviruses were initially grouped as a separate virus family on the basis of their distinct electron microscopic appearance. Although data on the antigenic interrelationships among the Coronaviridae are not yet complete, the viruses have been tentatively placed into four antigenic clusters (Table 1). These antigenic clusters consist of distinct and unrelated groups of avian and mammalian coronaviruses (71). Group 1 coronaviruses include the human coronavirus 229E (HCV-229E), transmissible gastroenteritis virus (TGE) of swine, canine coronavirus (CCV), and feline infectious peritonitis virus (FIPV). The group 1 coronaviruses are closely related antigenically and share common antigenic determinants on the nucleocapsid, and envelope glycoproteins (30,39,50). Coronaviruses in group 2 are antigenically unrelated to group 1 and include the human coronavirus OC-43 (HCV-OC43), several serotypes of MHV, BCV, and

TABLE 1. Antigenic cross-reactions between
coronaviruses

Mammalian

<u>Group 1</u>	<u>Group 2</u>
Human coronavirus-229E	Human coronavirus-OC43
Transmissible gastroenteritis	Mouse hepatitis
Canine coronavirus	Bovine coronavirus
Feline infectious peritonitis	Hemagglutinating encephalomyelitis

Avian

<u>Group 3</u>	<u>Group 4</u>
Infectious bronchitis virus	Turkey coronavirus

Taken from Wege (71).

hemagglutinating encephalomyelitis virus (HEV) of swine (71). Bovine coronavirus and HCV-OC43 are closely related antigenically within this group, but all members share common antigenic determinants (6,22,26). Groups 3 and 4 are the avian coronaviruses, avian infectious bronchitis virus (IBV) (39,71), and turkey coronavirus (TCV), respectively (71). These avian viruses do not share antigenic determinants with the mammalian coronaviruses or each other.

Bovine coronavirus was initially identified in fecal samples of calves with neonatal calf diarrhea (40,41). Neonatal calf diarrhea caused by BCV produces 90-100% morbidity and a mortality as high as 50% depending on conditions in the herd (4). Neonatal calf diarrhea is a problem of concern to producers of beef calves since late winter, early spring calving schedules expose neonatal calves to stressors (inclement weather), which increases susceptibility of calves to BCV (61). The incidence of BCV in beef and dairy calves has not been adequately assessed, but in one study in which fecal or ileal contents of 761 calves were examined by electron microscopy for BCV, BCV was present in 17% of the samples examined (47). Most of the calves determined to be shedding BCV in

their feces had diarrhea and some died from dehydration (47).

Most calves are infected with BCV at one day to 4 weeks of age (4,47,49). Gnotobiotic and colostrum-deprived calves develop diarrhea within nineteen to thirty hours after experimental inoculation with BCV (41,71). Diarrhea persists for three to five days and may result in severe dehydration and death or a permanent retardation of growth in calves, which survive the infection (46,71,72). The villous epithelial cells of the small intestine are the target cells for BCV. Virus replication occurs in the villous absorptive cells of the jejunum, the ileum, the colon, and the colonic crypt cells (41,46,71). Virus replication in the jejunum and ileum results in destruction of villous epithelial cells, resulting in villous atrophy, which decreases the absorptive surface of the small intestine, producing a malabsorptive diarrhea (41,46). The lesions in the colon are variable in severity and confined to the colonic crypt cells (46). Bovine coronavirus causes more severe and persistent lesions in the small intestine than those observed in calves inoculated with bovine rotavirus, which also produces diarrhea in calves (41,46).

Characterization of BCV depends on the ability to adapt the virus to replicate in cell culture. The addition of proteolytic enzymes such as trypsin to culture media significantly enhances virus growth in vitro (15). This result suggests that proteolytic enzymes in the intestinal tract may also promote or enhance the infectivity of BCV, but this hypothesis has not been adequately studied (71). Bovine coronavirus can be propagated on a continuous cell line of human rectal tumor (HRT-18 cells), derived from an adenocarcinoma (35). Electron microscopic studies of cell culture propagated BCV reveal that this virus possesses an additional layer of surface projections not observed on other coronaviruses (7,61).

Bovine coronavirus has five major structural proteins. Four of these proteins are glycosylated, partially external to the virion envelope, and have molecular weights of 120-140, 120, 100, and 26 kd (32). The fifth protein is the nucleocapsid protein, which is phosphorylated, located internally, and has a molecular weight of 52 kd (32). The 120-140 gp exists as a disulfide-linked dimer of two glycoproteins of 65 kd and functions as the virion hemagglutinin (6,32,33).

Treatment of BCV with bromelain or pronase

results in digestion-resistant 35 and 22 kd fragments that are located internally to the envelope of the virion (33). The 35 and 22 kd fragments represent portions of the surface projections and transmembrane glycoproteins.

Hemagglutinating encephalomyelitis virus. In 1957, a disease of nursing pigs characterized by high morbidity, vomiting, anorexia, constipation, and severe progressive emaciation was observed in Canadian swine herds (55). In the acute disease, vomiting and severe depression were the only clinical signs noted before death. The acute disease frequently progressed until affected suckling pigs became emaciated and died of starvation after a few weeks. Growth of surviving pigs was also premanently retarded. The condition was termed "vomiting and wasting disease" (55). Viral encephalomyelitis also appeared concurrently with the vomiting and wasting syndrome. The encephalomyelitis syndrome often progressed to produce muscle tremors, blindness, and paddling in the later stages of the disease prior to death (1,43). Viral encephalomyelitis was most common and severe in pigs under two weeks of age (1), but affected pigs up to four weeks of age (51). A hemagglutinating virus was isolated from

clinical specimens of pigs with vomiting and wasting disease and viral encephalomyelitis (23). Mengeling and Cutlip (43) were able to reproduce the central nervous system signs, and the vomiting and wasting syndrome using the same field isolates. The prototype field isolate designated as the 67N strain of HEV was isolated from the nasal cavity of an apparently healthy pig (43). Greig et. al. (23) also isolated a virus from brain tissue of pigs with encephalomyelitis and could experimentally reproduce the "vomiting and wasting" syndrome as well as encephalomyelitis in suckling pigs. The 67N strain of virus and the virus isolated and described by Greig et. al. (23) were determined to be identical, and the name hemagglutinating encephalomyelitis virus (HEV) was proposed, because the virus hemagglutinated red blood cells and caused encephalomyelitis in susceptible pigs. Pigs experimentally inoculated with HEV develop anorexia and vomition after a four to six day incubation period (3). The virus is apparently naturally transmitted through nasal secretions and the nasal passages are the primary site of replication. The virus multiplies in the epithelial cells of the nasal mucosa, tonsils, lungs, and small intestine (3). Following local

replication in and near the nasal passages, the virus spreads via the peripheral nervous system to the central nervous system and brain (2,3).

Hemagglutinating encephalomyelitis virus is antigenically similar to BCoV and also contains five structural proteins. Four of these proteins are glycosylated, partially external to the virion envelope and have molecular weights of 140-180, 120, 100, and 26.5 kD (8,52). The fifth protein is the nucleocapsid protein, which is phosphorylated, located internally, and has a molecular weight of 56 kD (52). The gp 140-180 exists as a disulfide-linked dimer of two glycoproteins of 76 kD and functions as the virion hemagglutinin (8). Thus, the hemagglutinin present on the HEV is very similar to the 120-140 kD glycoprotein hemagglutinin of BCoV (33).

Transmissible gastroenteritis (TGE) was first identified by Doyle and Hutching (18) and the etiological agent was later determined to be a coronavirus (39). Transmissible gastroenteritis is a highly contagious, enteric disease of swine characterized by a short incubation period (16-24 hrs), vomiting, severe diarrhea, and high mortality in piglets under two weeks of age (9,24,25,28). Mortality

in piglets under two weeks of age is usually 100 percent (24,25). Like BCoV, TGE replicates in and destroys the villous epithelial cells in the small intestine producing marked villous atrophy in the lower duodenum, jejunum, and ileum of pigs. This results in an acute malabsorptive diarrhea (28,29,45,48) followed by dehydration and death (28).

Transmissible gastroenteritis virus has a genome of 6.8×10^6 molecular weight (59) and three proteins of 200, 50, and 29 kd. The 50 kd protein is phosphorylated and internal to the virion envelope (6,20,21). The gp 29 is the transmembrane protein (36). The glycopolyptide of 200 kd is the peplomer (envelope) glycoprotein (20,21) and carries antigenic determinants responsible for the induction of neutralizing antibody (16,21,36). The gp 200 surface projection is also assumed to be the virus antireceptor, which binds to cellular receptor sites to initiate infection (21,36).

Canine coronavirus (CCV) and TGE have similar polypeptide structures and are closely related antigenically (20). Canine coronavirus was isolated from military dogs with diarrhea (5). The isolate, designated I-71, caused severe gastroenteritis in

puppies, but did not infect piglets (5,10). Acute enteritis and dehydration developed three to seven days after inoculation of susceptible puppies (5,31). The intestinal lesions were similar to lesions seen in pigs inoculated with TGE and were characterized by villous atrophy and fusion of intestinal villi throughout the small intestine, especially in the ileum (31). The intestinal villi regenerated within seven to ten days after infection and most dogs recover (5).

Serologically, CCV is more predominant among kennel dogs (62-87 percent) than among household dogs (22 percent) and the disease has a more severe course in very young compared to older puppies (71). Canine coronavirus has four structural proteins. Three are glycosylated, partially external to the virion envelope, and have molecular weights of 204, 32, and 22 kd (20). One species is the nucleocapsid protein which is internal to the virion envelope and is similar to the nucleocapsid protein of the other coronaviruses (20). Canine coronavirus may have two membrane proteins of 32 and 22 kd. Both of these proteins are glycosylated and similar to the transmembrane gp of other coronaviruses (20). The glycosylated protein of 204 kd is the envelope glycoprotein, which is similar

to the 200 kd gp of TGE (20). Presumably the 204 kd gp of CCV induces neutralizing antibody, but the functions of the proteins of CCV have not been adequately described and require further study.

Before the pioneering success of Kohler and Milstein (34) of immortalizing antibody secreting cells, use of antiviral antisera was always limited by the polyspecificity with regard to individual antigenic structures of a given viral protein (73). Production of monospecific antisera against individual viral proteins, particularly minor viral structural components, was difficult (73). The problems associated with the polyspecificity of antisera are avoided by the use of monoclonal antibodies. Monoclonal antibodies have defined specificities toward single antigenic determinants and provide potentially useful tools for the analysis of distinct structural domains of a protein (73). Due to the extreme specificity of monoclonal antibodies, it is possible to use them to define specific regions (epitopes) of a protein (66). Monoclonal antibodies represent a homogenous antibody population and thus achieve the highest degree of monospecificity possible for immunological reagents (73). It is possible to

identify a protein as performing a particular function, once an antibody is available which is capable of inhibiting that function (73). Monoclonal antibodies have been used to analyze the biological activities of murine coronavirus (71), bovine coronavirus (17,68), and several other coronaviruses (36,44).

The avian coronavirus, avian infectious bronchitis virus (IBV), has a surface projection gp S that is an oligomeric protein of two to three copies of each of two glycoproteins, S1 (90 kd) (11) and S2 (84 kd) (12). The S2 gp serves to anchor S while the S1 gp may form the major part of the bulbous distal end of the surface projection S (12). Avian infectious bronchitis virus, treated with urea to remove the S1 gp, is unable to induce virus neutralizing and hemagglutinating antibody. The S1 portion of the gp without the S2 portion present was able to induce these antibodies. This indicated that the S1 gp contained the appropriate epitopes for viral neutralization and hemagglutination and suggested that the epitopes for neutralization and hemagglutination were the same (13). This has been confirmed using monoclonal antibodies, which neutralize and prevent hemagglutination by IBV (44). These results suggest

that the hemagglutination and neutralization epitopes on the viral surface projections are either identical or in close proximity to each other such that antibody attaching to one antigenic site sterically alter the structure of the other antigenic site (44).

Like the other coronaviruses, mouse hepatitis virus (MHV) has a phosphorylated nucleocapsid protein of approximately 50 to 60 kd (14,62,63,65), and a transmembrane protein (gp 23) which has a pronase or bromelain resistant portion (63). The surface projection protein of 180 kd may undergo limited proteolytic cleavage yielding glycoproteins half the molecular weight or 90 kd (63). The surface projection protein is essential for virus attachment to receptors on the plasma membrane (27). Six different epitopic groups were found to be present on the surface of the MHV when a panel of monoclonal antibodies were studied. Some of the epitopes were responsible for neutralization of MHV and others caused fusion of cell membranes (70).

The gp 200 of TGE is similar to the gp 180 of MHV and induces neutralizing antibodies (16,20,36). The surface projection is also assumed to be involved with virus adsorption to the cell (20,36). Monoclonal

antibodies which neutralize TGE by binding to surface projections of the virus recognize four major antigenic sites or epitopes, which will induce neutralizing antibodies to this virus, while six epitopic groups have been reported for MHV (20,70).

A monoclonal antibody that neutralized BCV and inhibited BCV hemagglutination of rat erythrocytes immunoprecipitated the gp 140, or hemagglutinin of BCV (17). In contrast to these findings, Vautherot et. al. (68) reported two monoclonal antibodies which had neutralization and hemagglutination activities, but immunoprecipitated a glycoprotein of 105 kd rather than 140 kd as reported by Deregt (17). The results of Vautherot et. al. (68) are difficult to interpret since the gp 140 has been previously reported to be the hemaggultinin of BCV (33). Presently, the surface protein of BCV responsible for induction of neutralizing and hemagglutinating antibodies has not been identified. Monoclonal antibodies to BCV would provide a tool for further defining the biologically active regions of the BCV proteins and their role in pathogenesis of disease.

MATERIALS AND METHODS

Cells. Human rectal tumor (HRT-18) cells were obtained from Dr. G. N. Woode (Department of Veterinary Microbiology, Iowa State University, Ames, IA), and maintained on RPMI 1640 medium (Gibco Laboratories, Chagrin Falls, OH) as previously described (67). A continuous line of swine testicle cells (38) were obtained from the National Animal Disease Center, Ames, IA, and propagated in Eagle's basal medium (M. A. Bioproducts, Walkersville, MD) supplemented with 3% (v/v) lactalbumin hydrolysate. Crandell feline kidney (CRFK) cells were obtained from R. Crandell (Veterinary Medical Diagnostic Laboratory, Amarillo, TX) and maintained on Eagle's minimum essential medium (MEM) (Gibco Laboratories, Chagrin Falls, OH). All media were also supplemented with 200 µg/ml streptomycin, 400 U/ml of penicillin, 1 µg/ml fungizone, and 10% (v/v) irradiated fetal bovine serum (FBS) (Hyclone, Logan, UT) for growth media or 5% (v/v) FBS for maintenance media. Cells were maintained at 37 C in a humidified atmosphere of 5% CO₂.

Myeloma cell lines, P3-X63-Ag8.653 (ATCC, Rockville, MD); NS-1 (D. Gabrielson, North Dakota State

University, Fargo, ND); and SP2/OM1 (Salsbury Laboratories, Charles City, IA) were azaguanine-resistant derivatives of non-secreting lines of myeloma cell clones. These myeloma cell lines were maintained on Dulbecco's modification of Eagle's medium (Dulbecco's MEM) supplemented with 500 µg streptomycin, 1,000 U penicillin, 1% (v/v) Solution I, 1% (v/v) non-essential amino acids, and 10% (v/v) FBS. Solution I consisted of 100 ml of saline or distilled water containing 1,320 mg oxaloacetic acid, 80 mg insulin, and 550 mg sodium pyruvate (Sigma Chemical Co., St. Louis, MO). Myeloma cells were incubated at 37 C in a humidified atmosphere of 10% CO₂.

Viruses. The Nebraska strain of bovine coronavirus (BCV) and hemagglutinating encephalomyelitis virus (HEV 67N) were obtained from a commercial source (ATCC, Rockville, MD). The Purdue strain of transmissible gastroenteritis virus (TGE) was supplied by Dr. E. H. Bohl, Ohio Agricultural Research and Development Center, Wooster, OH. Canine coronavirus (CCV I-71) was obtained from Dr. L. Carmichael, Cornell University, Ithaca, NY. Bovine coronavirus was propagated on HRT-18 cells, HEV 67N, and TGE were grown on ST cells, and CCV I-71 was

propagated on CRFK cells.

Virus stocks were prepared by propagation of virus maintained on the appropriate cell line in serum-free media. When virus specific cytopathic effects were observed in 80% of the cell monolayer, cells were scraped from the flask, frozen, and thawed 3 times. Cell debris was removed by centrifugation at 1,240 x G. The virus containing pellets were resuspended in approximately 1/200 the original volume, dispensed into small aliquots, and stored at -70 C. These virus stocks were used in all assays in this study.

Production of hybridoma cell lines.

Eight-week-old Balb/c mice were inoculated intraperitoneally at 15 and eight days prior to fusion with 0.5 ml of BCV emulsified with an equal volume of Freund's complete adjuvant. Mice were again inoculated intraperitoneally with a suspension of BCV in saline at three, two, and one days prior to the harvest of spleen cells for production of hybridomas. Mice were also inoculated intravenously with a suspension of BCV in saline at two and one days prior to harvest of spleen cells.

Spleen cells from mice inoculated with BCV

were fused with myeloma cells using a modification of the procedure described by Galfre et. al. (19). Approximately 10^7 spleen cells from hyperimmunized mice were fused with 10^7 myeloma cells using 50% polyethylene glycol (M. W. 4000) (E. Merck, Darmstadt, Germany). Parental myeloma and spleen cells were eliminated by propagating the hybridoma cells in media containing hypoxanthine, aminopterin, and thymidine. Hybridomas were distributed into 24-well tissue culture plates and incubated until significant growth was apparent within two to three weeks after fusion. The culture fluids from each well of viable hybridomas were tested for antibody production by an enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IFA). Hybridomas which reacted with BCV on the ELISA or IFA assay were subcloned at least twice by limiting dilution in 96-well microtiter plates. Ascitic fluids were obtained by intraperitoneal injection of 10^6 to 10^7 hybridoma cells into Balb/c mice primed two to three weeks previously with Pristane-(2,6,10,14-tetramethylpentadecane, Sigma Chemical Co., St. Louis, MO). Culture and ascitic fluids were further screened for antibody reactivity to the coronaviruses used in this study by

hemagglutination inhibition, fluorescent focus neutralization, viral neutralization, and immune electron microscopy.

Enzyme-linked immunosorbent assay (ELISA) for monoclonal antibody to BCV. The ELISA was performed using a described technique (69). The ELISA antigens were produced by freezing and thawing BCV inoculated and uninoculated HRT-18 cells three times followed by centrifugation to remove cell debris. Immulon I microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with 100 μ l per well of a previously determined optimal dilution of BCV stock and HRT-18 host cell antigen in a carbonate-bicarbonate coating buffer (pH 9.6). The plates were incubated four hours at 37 C and stored at 4 C at least forty-eight hours before use. The coated plates were then washed three times in PBS (pH 7.2), containing 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO), and 0.2% (w/v) gelatin (Sigma Chemical Co., St. Louis, MO) before use.

One-hundred microliters of a 1:5 dilution of hybrid culture fluids were added to the BCV-coated plates and incubated for one hour at 37 C. Plates were then washed three times to remove the unbound antibody,

and bound antibody was detected by addition of 100 μ l per well of a 1:1,000 dilution of peroxidase-conjugated goat anti-mouse IgG (Cooper Biomedical, Malvern, PA). After incubation at 37 C for one hour, the plates were washed three times in PBS to remove the unbound conjugate and 100 μ l per well of substrate (2,2'-azino-di-(3-ethyl benzthiazaline-7-sulfonic acid, ABTS) in citric acid buffer (pH 4.0) was added to each well. Controls for the ELISA tests included HAT media containing FBS (control for antibody in FBS), rotavirus cell culture fluids from a hybridoma producing antibody to a porcine rotavirus (negative control), and polyclonal rabbit anti-BCV antiserum (positive control). After twenty minutes of incubation at room temperature, the absorbance of each well was recorded at dual wavelengths of 405/415 nm with a Titertek Multiskan spectrophotometer (Flow Laboratories, Inc., Rockville, MD).

Ascitic fluids were titered by ELISA using ten-fold dilutions of the monoclonal antibody. Ascitic fluids with titers lower than 1:1,000 were titrated using doubling dilutions. The titers were further defined by diluting the ascitic fluids in increments between the highest ELISA positive and negative

dilutions, i.e. ascitic fluid positive with an ELISA titer of 10^3 , but negative at 10^4 , was diluted to 10,000, 20,000, 30,000...90,000, 100,000 to determine the definitive titer. Optical density readings obtained for the normal host cell (HRT-18) antigen (background) were subtracted from the optical density readings of the positive ascitic fluids and the negative control samples. These data points were then graphed with the dilution of ascitic fluid on the x-axis and optical density on the y-axis. The best fit line for these data points was determined and extrapolated to determine where the line from data points of the positive ascitic fluid crossed the line from data points of the negative control. The ELISA titer of positive ascitic fluids was arbitrarily determined to be two dilutions prior to the point of crossover.

Isotyping. The isotype of each monoclonal antibody was determined using a commercial immunodiffusion test (Miles Laboratories, Naperville, IL).

Indirect immunofluorescence. Spot slides were prepared with cells infected with each coronavirus. Monolayers of the appropriate cell type were inoculated

as described in the section on viruses. When viral specific cytopathic effects were observed in 50% of the inoculated monolayer, a sterile bacteriological loop was used to scrape cells loose from the flask and transfer the cell suspension to wells on the spot slides. The virus-infected cell suspension was allowed to air dry and then fixed in acetone for ten minutes at room temperature. Slides were stored in plastic slide boxes at -70 C until used. Two-fold serial dilutions of ascitic fluids beginning at 1:10 were added to duplicate spots on the slides. The ascitic fluids that had a strong positive reaction were titered again using two-fold serial dilutions beginning at a 1:100 dilution. The slides were incubated for 30 minutes in a humidified chamber at 37 C, washed in PBS, and a 1:30 dilution of a fluorescein-isothiocyanate conjugated rabbit anti-mouse IgG (Cappell Laboratories, West Chester, PA), was added. After an additional 30 minute incubation at 37 C, slides were washed in PBS, rinsed in double-distilled water, and a coverslip was applied with mounting media (PBS-glycerol, pH 9.0) (Difco Laboratories, Detroit, MI). Slides were observed with a Zeiss epifluorescent microscope (Zeiss Invertoscope, Carl Zeiss, West Germany). The indirect

immunofluorescent titers of each tested ascitic fluid was the highest dilution at which fluorescence could still be observed.

Hemagglutination (HA) and hemagglutination inhibition (HAI) tests. These tests were done in 96-well "U-bottom" microtiter plates as previously described (23). All hybridomas were screened for inhibition of hemagglutination by BCV. The HA titer of the stock BCV was determined by adding 50 μ l of 1% washed rat erythrocytes to 50 μ l of a series of two-fold dilutions of BCV. The virus and red blood cells were incubated for one to two hours at ambient room temperature and the titer was expressed as the reciprocal of the highest dilution of BCV to cause complete agglutination of the rat erythrocytes. A dilution of BCV equivalent to four HA units was added to two-fold dilutions of each monoclonal antibody in sterile PBS. Plates were then incubated for thirty minutes at 37 C. Rat erythrocytes (1%) were then added to the virus-antibody mixture and incubated for one hour at room temperature. The titer of each monoclonal antibody was recorded as the reciprocal of the highest dilution capable of inhibiting hemagglutination of four HA units of BCV.

Viral neutralization (VN). Two-fold

dilutions, starting at a 1:4 dilution of ascitic fluids, were made in the appropriate media supplemented with 5% (v/v) FBS. An equal volume of media containing 100 TCID₅₀ of each coronavirus tested in this study was added to each well of a 96-well plate excluding the cell control. Cell controls contained no virus or ascitic fluid. Ascitic fluids which produced antibody specific for rotavirus were used as negative controls. The positive control was polyclonal rabbit anti-coronavirus serum for BCV, HEV, TGE, or CCV. Following a one hour incubation at 37 C, 100 µl of the appropriate cells were added to the virus-antibody mixture. Plates were incubated at 37 C for three to four days and each well observed for the presence or absence of specific viral cytopathic effect. Titers were expressed as the reciprocal of the highest dilution of antibody where no cytopathic effect was observed.

Fluorescent focus neutralization assay (FFN).

To determine the neutralizing antibody titer of each monoclonal antibody, equal volumes of two-fold serial dilutions of the antibody and BCV were mixed and allowed to react at 37 C for one hour. The stock BCV

was used at a dilution previously determined to produce 100 fluorescent cells per well. The antibody-virus mixture was then added to 96-well plates of washed HRT-18 monolayers. After a one hour incubation at 37 C, the inoculum was removed and 200 μ l of serum-free media was added to each well. Plates were then incubated at 37 C for 48 hours. At the end of the incubation period, cells on the plates were fixed with 60% acetone and a 1:20 dilution of polyclonal rabbit anti-BCV serum was added to each well and incubated at 37 C for 30 minutes. The excess serum was washed off and a fluorescein-isothiocyanate conjugated goat anti-rabbit IgG was added to each well. Plates were incubated at 37 C for 30 minutes, washed in PBS, and 100 μ l of PBS was added to each well before observation. Plates were observed with an epifluorescent microscope as previously described in the section on indirect immunofluorescence. The neutralizing antibody titer was expressed as the reciprocal of the highest dilution of serum that reduced fluorescence by 80% compared to the virus control wells.

Bromelain treatment of BCV. Bromelain digestion of the outer glycoprotein peplomers of BCV

was done as previously described (8,20,51,52). One volume of a bromelain solution consisting of 1.3 mg/ml, 0.001 M EDTA, and 0.05 M dithiothreitol (Sigma Chemical Co., St. Louis, MO) was added to nine volumes of BCV. The suspension was incubated at 37 C for two hours and then centrifuged at 40,000 x g for 30 minutes. The supernatant was discarded and the virus pellet was resuspended in Hank's balanced salt solution for use in immune electron microscopy.

Immune electron microscopy (IEM). A modification of the procedure of Saif et al (56) was used. Equal volumes of untreated or bromelain treated BCV and ascitic fluids from each clone were mixed and incubated at 37 C for one hour, then held overnight at 4 C. The suspension was then centrifuged at 40,000 x g for 45 minutes to pellet virus-antibody complexes. The supernatant was discarded and the pellet was resuspended in one drop of 4% phosphotungstic acid (PTA) pH 6.5, two drops of 0.01% bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St. Louis, MO), and double-distilled water. The suspension was sprayed with a globuonebulizer onto collodion and carbon coated grids and examined on a Hitachi HU-12A (Hitachi Ltd., Japan) electron microscope. Controls included

untreated and bromelain treated BCV mixed with either negative ascitic fluid or polyclonal rabbit anti-BCV antiserum as well as bromelain treated and untreated BCV.

RESULTS

Seven hybridomas from two separate fusions were found to secrete monoclonal antibodies to BCV, when screened by the ELISA and IFA procedures. The biological and antigenic specificities of the seven BCV hybridomas are summarized in Table 2. Monoclonal antibodies of the IgG₁ isotype were most common.

Each of the seven hybridomas secreting monoclonal antibody to BCV gave a positive reaction on the indirect fluorescent antibody test with BCV and 3/7 (43%) monoclonal antibodies also reacted with HEV. No membrane fluorescence was observed. Cytopathic fluorescence varied from a fine granular fluorescence to diffuse or globular fluorescence (Fig. 1). Immuno-fluorescent titers against BCV ranged from 1:10 to 1:3,200. The three hybridomas (71-44, 6-1, 6-4) which also reacted with HEV, had higher titers to the homologous (BCV) compared to the heterologous (HEV) antigen. The IFA titers were five to 20 times higher when BCV rather than HEV was used as antigen. Immunofluorescent titers against BCV ranged from 1:10 to 1:3,200. The hybridomas did not produce antibody which reacted on IFA against CCV and TGE infected

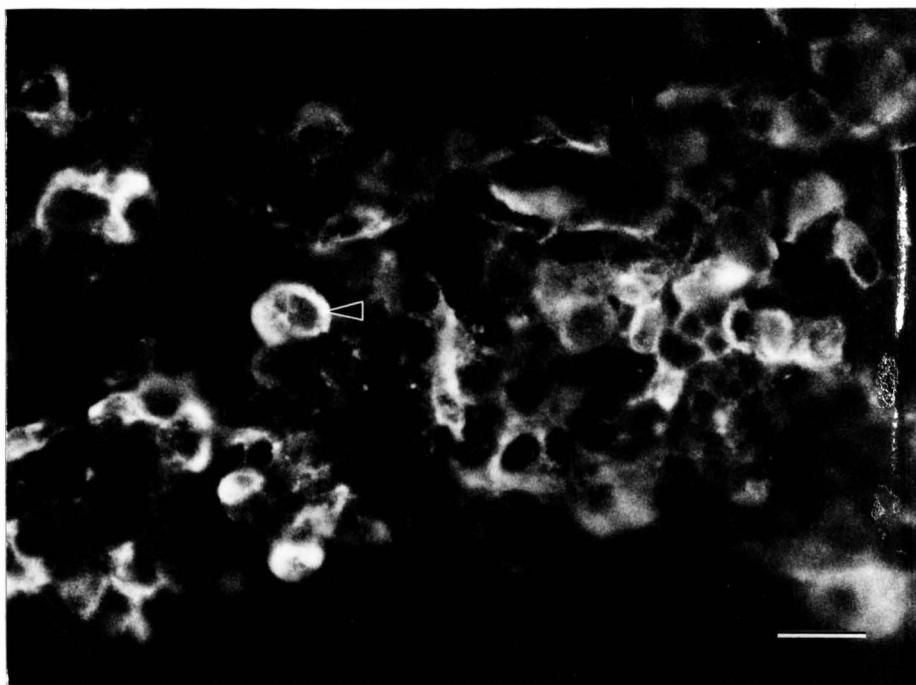
TABLE 2. Characterization of anti-BCV monoclonal antibodies

<u>Designation</u>	<u>Isotype</u>	<u>IFA titer²</u>		<u>ELISA titer³</u>
		<u>BCV</u>	<u>HEV</u>	<u>BCV</u>
71-44	IgG ₁	1:1,600	1:80	1:150,000
88-29	IgG _{2a}	1:10	<10	1:40
25-48 ¹	IgG ₁	1:3,200	<10	Negative
4-2	IgG ₁	1:400	<10	1:900,000
4-7	IgG ₁	1:100	<10	1:80,000
6-1	IgG ₁	1:800	1:160	1:1,000,000
6-4	IgG ₁	1:800	1:160	1:900,000

¹ Clone 25-48 also neutralized BCV.

² IFA = indirect immunofluorescence; BCV = bovine coronavirus; HEV = hemagglutinating encephalomyelitis. No reaction was observed by IFA when transmissible gastroenteritis virus and canine coronavirus were used as antigens. The IFA titers were expressed as the reciprocal of the highest dilution where fluorescence was still observed.

³ ELISA = enzyme-linked immunosorbent assay. Titers were determined as detailed in Materials and Methods.



cells.

The ELISA titers of the ascitic fluids ranged from 1:40 to 1:1,000,000 as indicated by Table 2 and Figs. 2, 3, and 4. The titer of each monoclonal antibody on the ELISA was determined by estimating where the best fit line of the positive data crossed the line from the data points of the negative control. Two dilutions prior to the last positive dilution was arbitrarily reported as the titer of each antibody. The ELISA titers for 4-2, 4-7, 6-1, and 6-4 were 2,250; 80; 1,250; and 1,125 times greater than the titers on IFA, respectively. The ELISA titer for 71-44 was 100 times greater than the IFA titer against BCV and 88-29 had an ELISA titer four-fold greater than the IFA titer. However, 25-48, which had the highest IFA titer, did not react with BCV antigen on ELISA.

The biological activities of monoclonal antibodies were also assayed by HAI and viral neutralization. Only 1/7 (14%) of the clones (clone 4-7) inhibited hemagglutination of BCV at a low titer of 1:16. In the conventional viral neutralization testing, doubling dilutions of the ascitic fluid was allowed to react with 100 TCID₅₀ of BCV, HEV, TGE, and CCV, and none of the seven hybridomas neutralized any

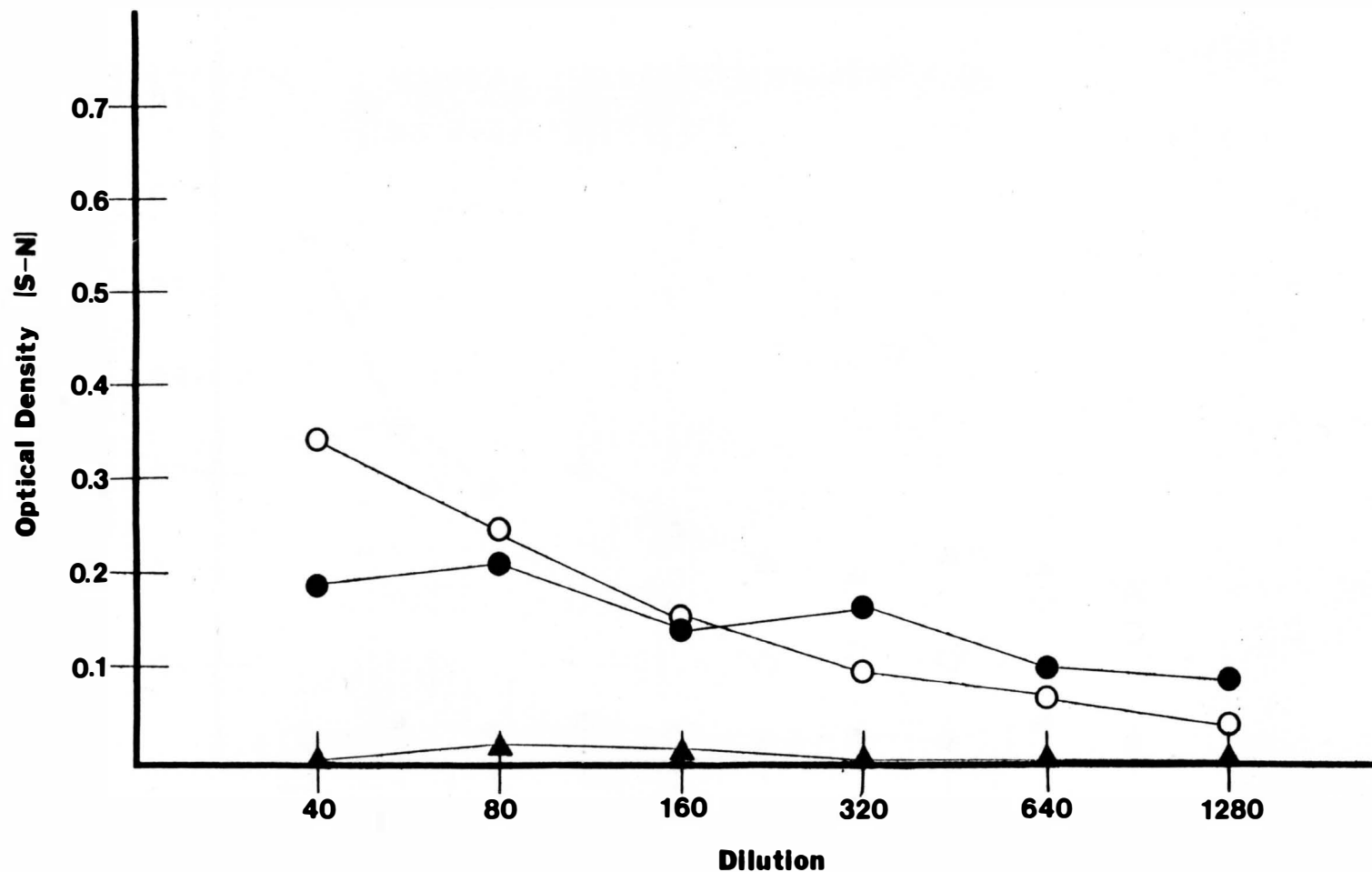


Fig. 2. The ELISA titers of ascitic fluids 25-48 (▲), 88-29 (○), and negative control (●). The optical density (S-N) is expressed as the optical density of the reaction between ascitic fluid and BCV (S) and the optical density of the reaction between ascitic fluid and the normal host cell (N).

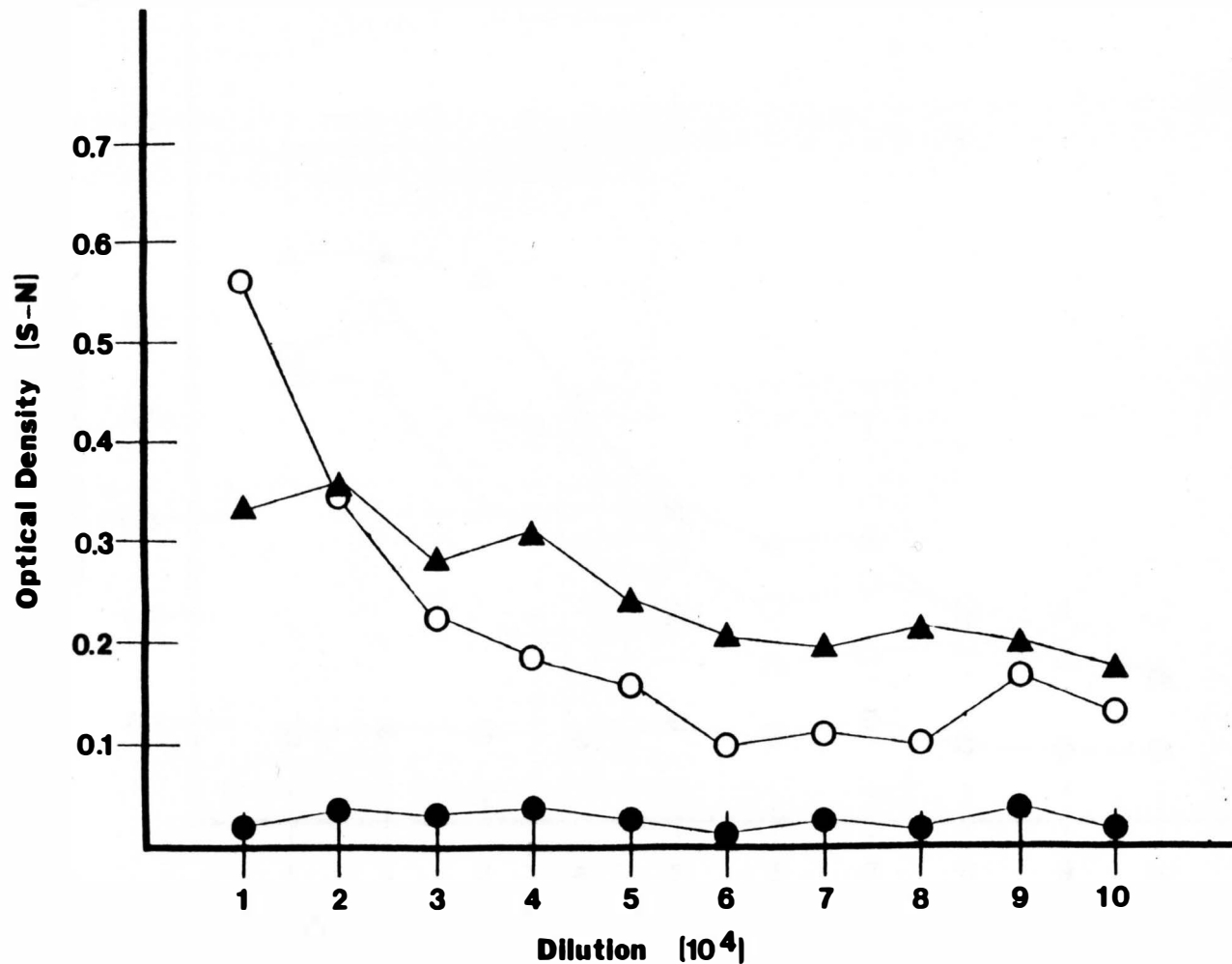


Fig. 3. The ELISA titers of ascitic fluids 71-44 (▲), 4-7 (○), and negative control (●). The optical density (S-N) is expressed as the optical density of the reaction between ascitic fluid and BCV (S) and the optical density of the reaction between ascitic fluid and the normal host cell (N).

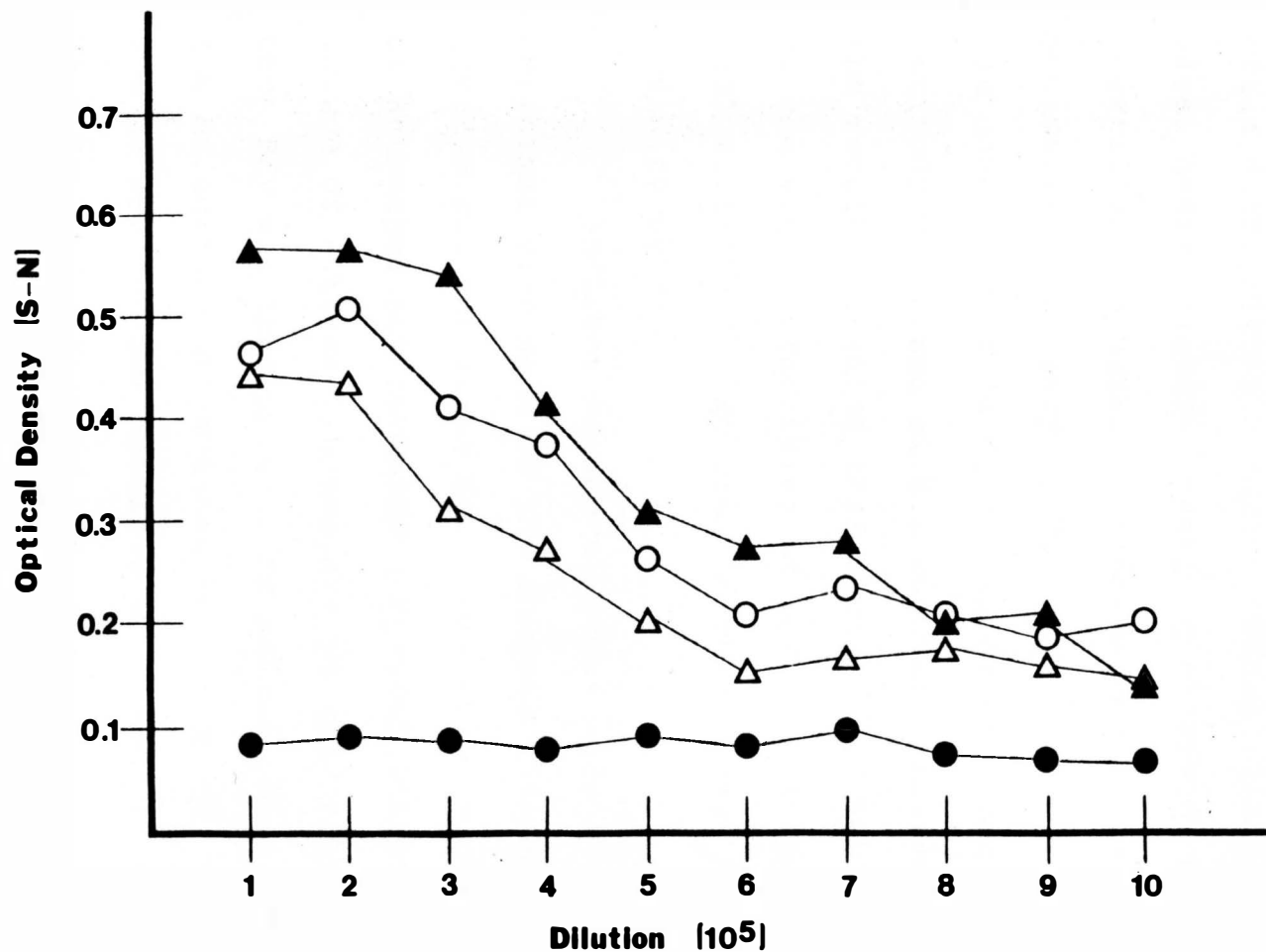
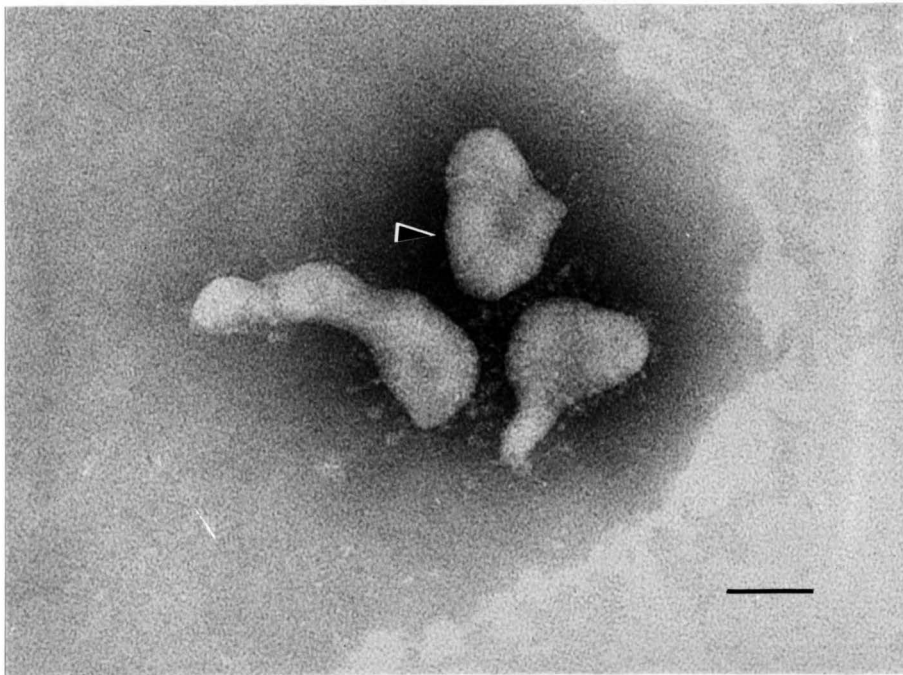
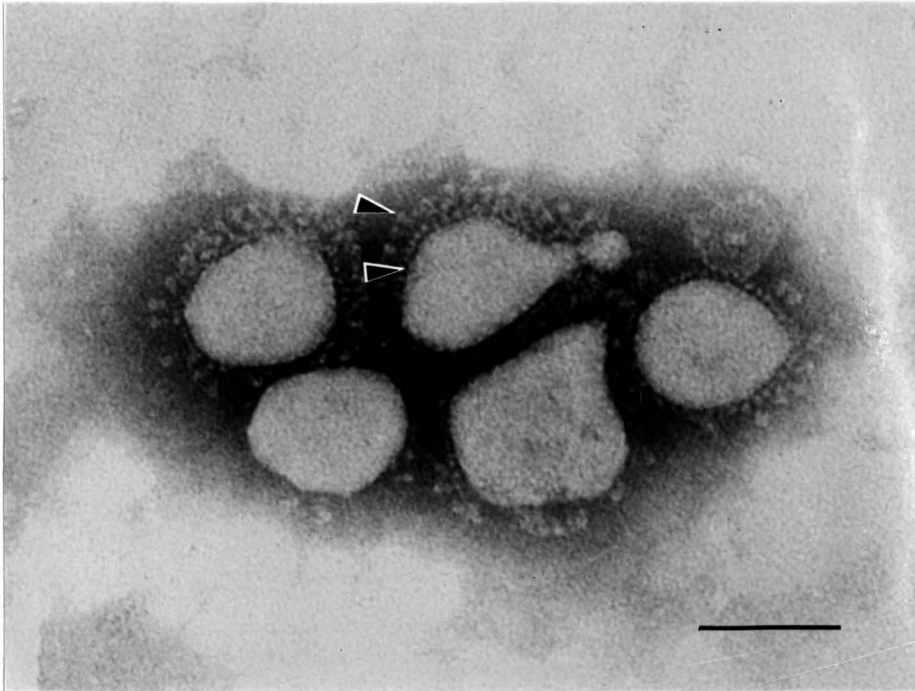


Fig. 4. The ELISA titers of ascitic fluids 6-4 (▲), 4-2 (○), 6-1 (△), and negative control (●). The optical density (S-N) is expressed as the optical density of the reaction between ascitic fluid and BCV (S) and the optical density of the reaction between ascitic fluid and the normal host cell (N).

of the four viruses. However, it was observed that the cytopathic effect (CPE) of BCV on HRT-18 cells was often difficult to observe. Since this influenced the viral neutralization assay, an alternative neutralization assay, a fluorescent focus neutralization assay (FFN) which does not rely on CPE, was done. Two-fold dilutions of each monoclonal antibody was mixed with a constant dilution of BCV and the results revealed that clone 25-48 neutralized 80% of the viral infectivity of BCV at a titer ranging from 1:32 to 1:1,024. No other clones showed neutralizing activity by FFN.

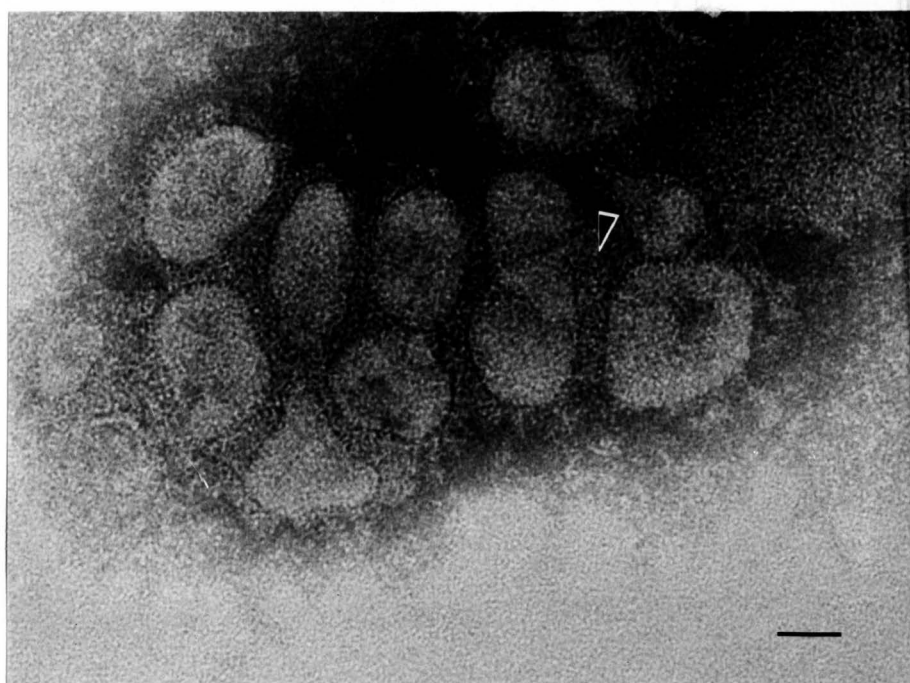
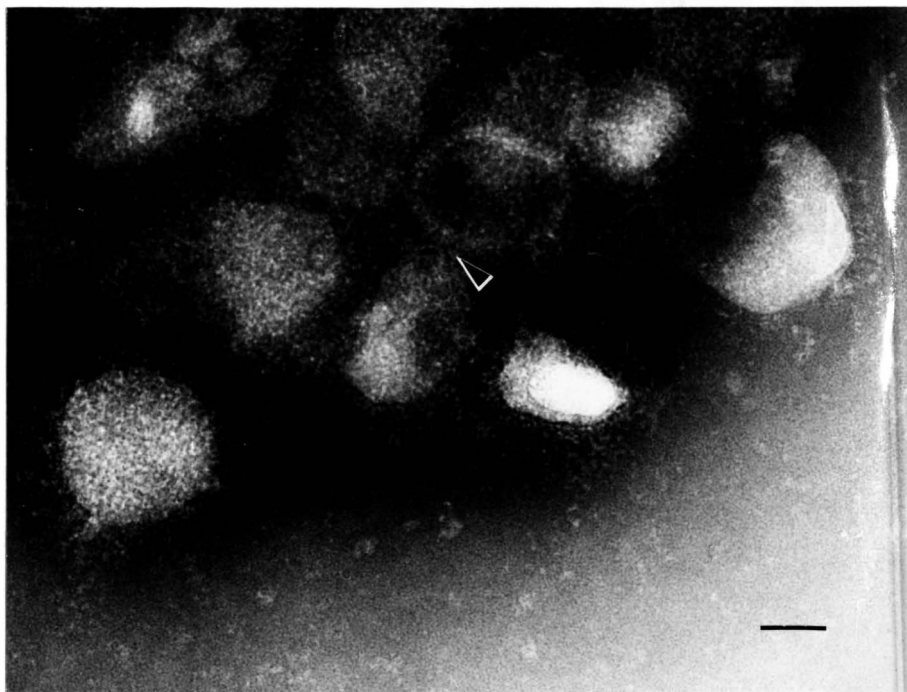
Indirect immune electron microscopy was performed with each of the ascitic fluids and bromelain treated and untreated BCV. Direct electron microscopy of untreated BCV revealed a typical BCV with a double fringe of surface projections as indicated in Fig. 5a. When BCV was treated with bromelain, most of the outer row of surface projections were removed, but the inner row of projections appeared to remain intact (Fig. 5b). When bromelain treated BCV was incubated with a negative ascites control, the inner row of surface projections and a few remaining surface projections of the outer row were easily observed indicating that no

Figure 5. (a) Normal BCV morphology with two rows of surface projections as indicted by arrows (Bar = 50 nm); (b) Treatment of BCV with bromelain results in loss of the outer row of surface projections (Bar = 50 nm).



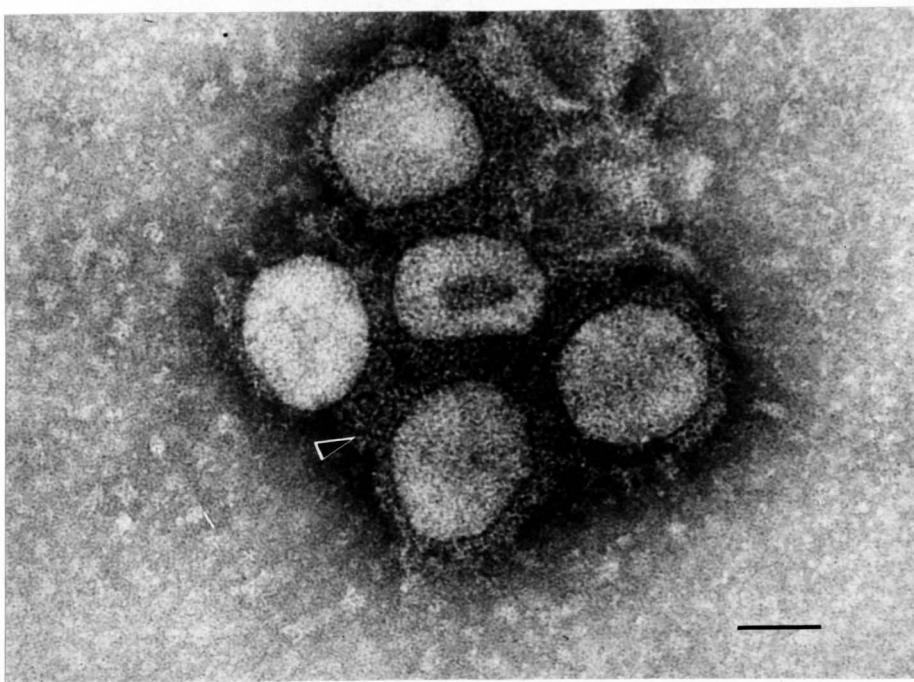
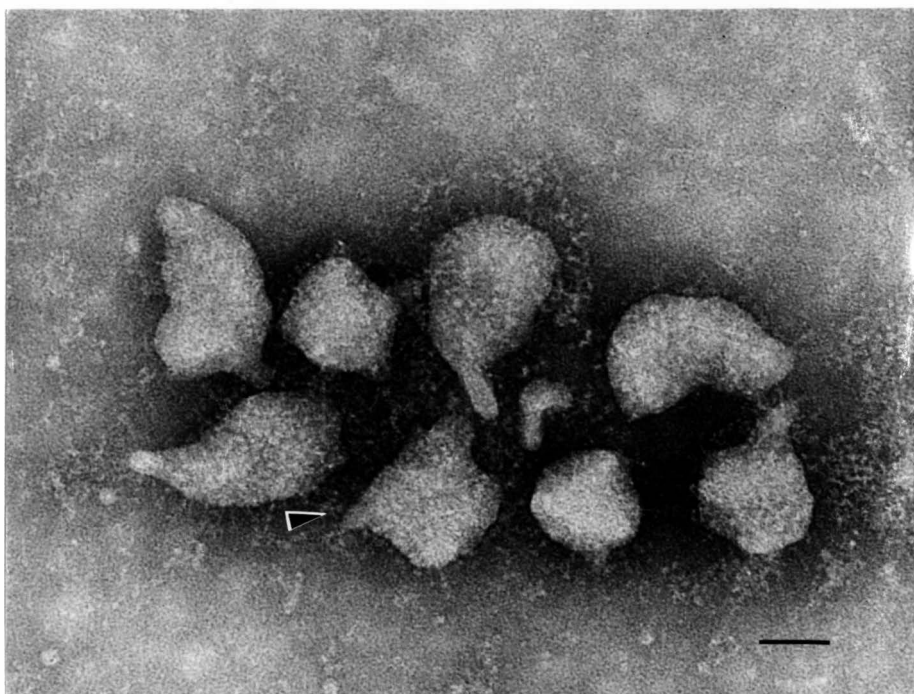
antigen-antibody reaction took place. As a positive control, BCV was incubated with polyclonal rabbit anti-BCV serum (Fig. 6a). This resulted in aggregation of virus particles, 20 to 30 viral particles were observed per aggregate, and the viral morphology was obscured by antibody bound to the surface of the viral particle. When bromelain treated BCV was incubated with polyclonal rabbit anti-BCV serum, there appeared to be more antibody attached to the treated virus particles compared to the untreated virus (Fig. 6b). The morphologic detail of the surface projections on the bromelain treated BCV compared to untreated BCV particles was more difficult to observe due to the increased amount of antibody, bound to the treated virus. Although more antibody bound to treated BCV compared to untreated BCV, increased aggregation of particles or an increase in the number of virus particles in the virus-antibody aggregates was not observed. The surface projections of BCV were partially obscured when untreated BCV was incubated with ascitic fluid 25-48 which neutralized BCV on the FFN assay (Fig. 7a). When bromelain treated BCV was incubated with ascitic fluid from 25-48, the reaction of the monoclonal antibody and BCV was similar to that

Figure 6. (a) Untreated BCV incubated with polyclonal rabbit anti-BCV antiserum (positive control) shows the BCV particles aggregated in large clumps with surface projections obscured as indicated by arrow (Bar = 50 nm); (b) Bromelain treated BCV incubated with polyclonal rabbit anti-BCV antiserum shows larger amounts of antibody bound to the treated compared to untreated BCV in Fig. 6a. Peplomers obscured by antibody are indicated by arrow (Bar = 50 nm).



described for the reaction between the bromelain treated BCV and polyclonal rabbit anti-BCV serum (Fig. 7b). There was increased binding of antibody observed when BCV was reacted with either 25-48 or polyclonal rabbit anti-BCV serum for bromelain treated BCV. Antibody surrounded BCV particles and surface projections were indistinguishable from antibody. Ascitic fluid from hybridomas 71-44, 88-29, 4-2, 4-7, 6-1, and 6-4 did not bind to the surface of BCV. The hybridomas were not tested with HEV, TGE, and CCV by IEM since no neutralization was obtained in the viral neutralization assays.

Figure 7. (a) Untreated BCV incubated with ascitic fluid 25-48 shows monoclonal antibody bound to the surface projections of the aggregated particles as indicated by arrow (Bar 50 nm); (b) Bromelain treated BCV incubated with the neutralizing monoclonal antibody 24-48 shows markedly greater amounts of antibody bound to the surface (as indicated by arrow) when compared to untreated BCV incubated with ascitic fluid 25-48. The surface projections are greatly obscured by monoclonal antibody and a larger amount of antibody is bound to the aggregated virus particles (Bar = 50 nm).



DISCUSSION

The objective of the present study was to develop and characterize a panel of monoclonal antibodies to the Nebraska strain of bovine coronavirus. Monoclonal antibodies can be used to associate viral polypeptides with biological functions such as viral attachment, neutralization, hemagglutination, and cell fusion. Monoclonal antibodies have been used to characterize the structural polypeptides of avian (IBV) (44), porcine (TGE) (16,36), murine (MHV) (14,70), and bovine (BCV) (17,68) coronaviruses. There are only two reports in the literature describing the use of monoclonal antibodies to determine the biological functions of the five to seven structural polypeptides of BCV (17,68). Current evidence suggests the hemagglutinating and neutralization epitopes on the surface of BCV may either be similar (17,68), or different (68). Development of monoclonal antibodies to the hemagglutinin or neutralization epitopes of BCV would be important not only in identification of structural proteins of the virus, but also could be used in hemagglutination inhibition or neutralization

tests for detection of antigen in clinical material or to determine the degree of antigenic variation among different isolates of BCV. Although BCV is presumed to be a single serotype, two monoclonal antibodies directed to the French strain of BCV failed to cross-react with either the United States or British isolates of BCV (68). In the present study, seven monoclonal antibodies to bovine coronavirus were characterized by biological assays to determine their reactivity with bovine coronavirus, transmissible gastroenteritis virus, hemagglutinating encephalomyelitis virus, and canine coronavirus. Of the seven monoclonal antibodies characterized, 6/7 reacted on the ELISA assay, only the neutralizing monoclonal antibody (25-48) could not be detected using the ELISA. All monoclonal antibodies reacted with BCV on IFA and 3/7 also cross-reacted with HEV. One monoclonal antibody (4-7) inhibited viral hemagglutination, but did not neutralize BCV, suggesting that the hemagglutinin and neutralization epitopes are either on different glycoproteins or different locations on the same glycoprotein. The latter hypothesis was confirmed by immune electron microscopy, since monoclonal antibody from clone 25-48

neutralized and reacted with bromelain treated BCV.

The enzyme-linked immunosorbent assay was used as the screening assay to determine if hybridomas secreted antibody specific to BCV. The ELISA is a highly sensitive assay (54,73) that is independent of monoclonal antibody isotype, and suitable for rapid assessment of the specificity of the hybridoma antibodies (73). This assay is dependent on the ability of proteins (antibodies or viral proteins) to adhere to a solid phase, which is usually a plastic microtiter plate (53). The binding of proteins to the plastic is pH and temperature dependent (53). In the ELISA used in this study, BCV was bound to plastic microtiter plates. Culture supernatant from 7/7 clones and ascitic fluid from 6/7 clones reacted with BCV bound to the microtiter plates. Only ascitic fluid from clone 25-48, which neutralized BCV, did not react on ELISA. This result was not expected because culture supernatant taken from hybridoma culture 25-48 prior to cloning did react on the ELISA. The lack of reactivity of ascitic fluid 25-48 on the ELISA could be explained if: 1) the monoclonal antibody degraded upon binding to BCV, adhered to the solid phase, 2) the avidity (binding affinity) of the monoclonal antibody to BCV

was weak and washing may have removed antibody bound to BCV, and 3) the viral epitopes recognized by the monoclonal antibody were modified, degraded, or masked when BCV was bound to the solid phase. Monoclonal antibodies which neutralize porcine rotavirus have also been reported to react poorly or not at all on ELISA (Benfield, personal communication). The ELISA test was the most sensitive serological assay for determining antibody because titers were generally 80 to 2,250 times higher than titers obtained with the IFA. The higher antibody titers obtained with the ELISA compared to IFA confirms previous reports that the ELISA is the most sensitive method to detect most monoclonal antibodies specific to BCV (68). The monoclonal antibodies that react with BCV in the ELISA test could be used to develop an antigen capture ELISA to detect BCV in fecal material of infected calves.

Although the IFA is not as sensitive an assay as the ELISA, ascitic fluid derived from the six nonneutralizing and the one neutralizing hybridomas did react with antigen in BCV infected HRT-18 cells. The finding that 3/7 monoclonal antibodies (71-44, 6-1, and 6-4) also reacted with antigen in HEV infected cells confirms previous reports that BCV and HEV share common

antigens (6,71). There are also proteins common to BCV which are not present in HEV since 4/7 nonneutralizing monoclonal antibodies reacted only with BCV infected cells. Bovine coronavirus and HEV are in the same antigenic grouping, whereas TGE and CCV are in a different antigenic grouping of coronavirus (6,71). Thus, we did not expect any of the monoclonal antibodies to react with TGE or CCV and this expected result was observed.

Although BCV and HEV have been reported to be antigenically similar, most of these studies were done using polyclonal heterotypic serum and there was considerable ambiguity in results. The widespread occurrence of BCV-neutralizing antibodies in "normal" human and animal sera (22,57) inhibits the use of such antisera in studies of antigenic interrelationships. The anti-BCV antibodies present in human or animal sera could result from a past infection with BCV or a related coronavirus. The use of monoclonal antibodies, which are homogenous and monospecific, avoids the problems of cross-reacting antibodies present in polyclonal antisera. Thus, our data represents the first report that monoclonal antibodies recognize a common epitope present on BCV and HEV. These three

monoclonal antibodies probably react with an internal virion protein, nucleocapsid, or transmembrane proteins, since these monoclonal antibodies did not prevent hemagglutination or neutralization and were not observed by electron microscopy to bind to the virion surface. Previous reports indicate that the antigenic sites responsible for induction of neutralizing antibodies are associated with the surface glycoproteins (71) and the virion hemagglutinin of BCV is gp 140, an external protein (33).

Coronavirus glycoproteins are present on the surface of the virus and function in inhibition of hemagglutination (13,32) neutralization (13,14), cell attachment (16,27,36), and cell fusion (14). In this study, clone (25-48) neutralized BCV and one other clone (4-7 prevented hemagglutination of rat red blood cells by BCV. The monoclonal antibodies derived in this study were specific to BCV, since the other coronaviruses that were tested in the same or different antigenic groupings were not neutralized. The derivation of monoclonal antibodies which recognize separate epitopes, one which results in viral neutralization and one which prevents hemagglutination, would suggest that the viral glycoproteins containing

the neutralization site and the hemagglutinin are separate glycoproteins. Alternatively, the two monoclonal antibodies may recognize separate epitopes on the same glycoprotein and this glycoprotein may be the site of both neutralization and the viral hemagglutinin.

Previous reports have demonstrated that BCV has four surface glycoproteins of 140, 120, 100, and 26 kd molecular weight (32). Bromelain digestion of BCV removes all glycoproteins, except gp 140, which is the viral hemagglutinin (33). The gp 140 is the inner row of surface projections observed on electron microscopy in this study and by others (32). Bromelain digestion of BCV resulted in enhanced binding of the neutralizing monoclonal antibody 25-48 to the virion. This suggests that 25-48 binds to the gp 140, which is reported to be the only glycoprotein resistant to bromelain treatment. Since bromelain treatment enhanced antibody binding to BCV, the epitope recognized by 25-48 may be partially masked by other surface glycoproteins which are removed by bromelain treatment. No previous studies using monoclonal antibodies to BCV and immune electron microscopy techniques have been reported, but it has been determined that bromelain treatment of BCV will

also markedly enhance viral hemagglutination (33). While our observations that monoclonal antibody 25-48 binds to epitopes on the gp 140 is circumstantial, the results of the immune electron microscopy studies and bromelain digestion of BCV support the previous observations of King et. al. (33) that the gp 140 is resistant to bromelain digestion and is present on the inner row of surface projections of the virion. However, direct substantiation of the hypothesis that monoclonal antibody 25-48 binds to the gp 140 will require Western blotting and immunoprecipitation techniques.

Although Western blotting and immunoprecipitation were not done in this study, monoclonal antibodies, which both neutralize and inhibit hemagglutination by BCV have been reported to precipitate a gp 120 (17), which is analogous to the gp 140 (hemagglutinin) described by King et. al. (33). This suggests, as do our results, that the site of neutralization and hemagglutination co-exist on the same glycoprotein. In addition, Deregt et. al. (17) reported that one neutralizing monoclonal antibody, which did not hemagglutinate, also precipitated gp 120, further substantiating the possibility that there are

separate neutralization and hemagglutination epitopes on the gp 120-140 hemagglutinin.

Monoclonal antibodies which neutralize the avian coronavirus IBV also prevent hemagglutination indicating that epitopes for both functions are located on the same glycoprotein (12). However, IBV does not have a gp 120-140 dimer common to BCoV and it is possible that the neutralization and hemagglutination sites on IBV may be separate epitopes on the same glycoprotein. This latter alternative was suggested by Mockett et. al. (44). Since the neutralization and hemagglutination sites are in close proximity to one another, neutralizing antibody attaching to one site may sterically hinder the binding of red blood cells to the hemagglutinin.

The results of this and previous studies (17,33) disagreed with Vautherot et. al. (68), who reported that epitopes for both neutralization and hemagglutination resided on gp 105, which is analogous to the gp 120 described by King et. al. (32). The gp 105 or 120 are present on the outer row of surface projections and can be removed by bromelain digestion. All of the monoclonal antibodies which neutralized or inhibited hemagglutination, described by Vautherot et.

al. (68) also immunoprecipitated gp 105. However, it would be interesting to determine if the monoclonal antibodies described by Vautherot et. al. (68) actually bind to bromelain treated virus, which would be devoid of gp 105. Unfortunately, our monoclonal antibody, which prevented hemagglutination (4-7) did not bind to BCV on immune electron microscopy. Since the hemagglutination inhibition titer of the ascitic fluid was low (1:16), this antibody may have a low binding affinity for the hemagglutinin. Therefore, the minute amount of antibody bound could not be visualized by electron microscopy. Since this antibody inhibited hemagglutination, it is assumed that it recognizes an epitope on gp 140 or that the outer layer of surface projections may have sterically interfered with the binding.

In conclusion, seven monoclonal antibodies were characterized to determine specificity for epitopes on the Nebraska strain of BCV. Only clone 25-48 neutralized BCV. No other clones neutralized BCV, HEV, TGE, or CCV. Results of this study using a neutralizing monoclonal antibody support previous reports that the neutralization and hemagglutination function of the virus reside on the same glycoprotein

(13,17). Results from the IEM study using the neutralizing monoclonal antibody and bromelain treated virus provide evidence to substantiate the previous report (17) that gp 140, which is the viral hemagglutinin, is also the site of neutralization.

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